

## ORIGINAL ARTICLE

# Compound heterozygous null mutations of *NOBOX* in sisters with delayed puberty and primary amenorrhea

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## Abstract

**Background:** Premature ovarian insufficiency (POI) is a heterogeneous clinical syndrome defined by a premature loss of ovarian function that associates menstrual disturbances and hypergonadotropic hypogonadism. POI is a major cause of female infertility affecting 1% of women before the age of 40 and up to 0.01% before the age of 20. The etiology of POI may be iatrogenic, auto-immune or genetic but remains however undetermined in a large majority of cases. An underlying genetic etiology has to be searched in idiopathic cases, particularly in the context of a family history of POI.

**Methods:** Whole exome sequencing (WES) was performed in trio in a Belgian patient presenting POI and in her two parents. The patient presented delayed puberty and primary amenorrhea with hypergonadotropic hypogonadism.

**Results:** WES identified two novel compound heterozygous truncating mutations in the Newborn oogenesis homeobox (*NOBOX*) gene, c.826C>T (p.(Arg276Ter)) and c.1421del (p.(Gly474AlafsTer76)). Both mutations were confirmed by Sanger sequencing in the proband's sister who presented the same phenotype. Both variants were pathogenic and very likely responsible for the severe POI in this family.

**Conclusion:** We report here for the first time compound heterozygous truncating mutations of *NOBOX* in outbred patients, generalizing biallelic *NOBOX* null mutations as a cause of severe POI with primary amenorrhea. In addition, our findings also suggest that *NOBOX* haploinsufficiency is tolerated.

## KEYWORDS

delayed puberty, genetics, loss of function, next generation sequencing, *NOBOX* gene, premature ovarian insufficiency

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## 1 | INTRODUCTION

Premature ovarian insufficiency (POI) encompasses a broad spectrum of conditions associated with a highly heterogeneous clinical presentation ranging from ovarian dysgenesis with delayed puberty and primary amenorrhea to secondary amenorrhea. According to the ESHRE guidelines, the diagnosis of POI is based on the presence of amenorrhea or oligoamenorrhea before the age of 40 for at least 4 months, associated to hypergonadotropic hypogonadism with FSH levels above 25 IU/L on two occasions more than 4 weeks apart (Webber et al., 2016). POI affects ~1% of women before the age of 40 and up to 0.01% before the age of 20 (Webber et al., 2016). The etiology of POI may be iatrogenic, auto-immune or genetic. More recently, environmental pollutants and infectious agents have also been incriminated in POI development (Dutta et al., 2017; Nilsson et al., 2018; Vabre et al., 2017). Established genetic causes of POI include chromosomal abnormalities, mainly X chromosome numerical and/or structural defects, *FMR1* (OMIM number: \*309550) premutations and much rarer gene mutations involved in follicular development, folliculogenesis and ovarian aging (Ebrahimi & Akbari, 2015; Huhtaniemi et al., 2018; Pelosi et al., 2015; Qin et al., 2015; Tucker et al., 2016). A family history of POI and/or early menopause is reported in up to 31% of POI cases suggesting a significant underlying genetic component (Jiao et al., 2018; Vegetti et al., 1998). However, the etiology remains undetermined in a large majority of cases (Jiao et al., 2018). Identifying the genetic factors implicated in the pathophysiology of POI is of utmost importance to gain more insight in the molecular mechanisms responsible for the development of the syndrome. It can also contribute to improve genetic and reproductive counselling in patients and their relatives who, if diagnosed at risk through a genetic screening, can be offered fertility preservation before the exhaustion of their ovarian reserve. POI clinical presentations are very heterogeneous, even within a family suggesting a variable expressivity which could be related to other genes or epigenetic factors modulating the age of POI onset and/or the severity of clinical presentation (Bouilly et al., 2016; Hyon et al., 2016). Several genomic approaches have been used in the last decades

to identify genetic factors implicated in POI, including candidate gene approaches, linkage studies in multiplex families, whole genome association studies (GWAS), cytogenomic studies and more recently next generation sequencing (NGS) techniques which turned out to be powerful tools and contributed to the large expansion of causal or candidate gene variants in affected patients (Alvaro Mercadal et al., 2015; Jolly et al., 2019; Laissue, 2018; Qin et al., 2015; Sassi et al., 2020; Tiotiu et al., 2010; Tucker et al., 2018, 2019). These genes have been shown to be implicated directly or indirectly in gonadal and follicular development through very different mechanisms including mitochondrial and immune function, metabolism, apoptosis, DNA replication and repair, mRNA processing, cell cycle progression, meiosis and hormonal signaling (Huhtaniemi et al., 2018; Tucker et al., 2016).

In this study, we used whole exome sequencing (WES) to identify a genetic etiology of POI in two sisters from an outbred Belgian family presenting delayed puberty and primary amenorrhea (PA) with hypergonadotropic hypogonadism. We identified two novel, compound heterozygous, truncating mutations in the *NOBOX* gene (OMIM number: \*610934). Both variants were pathogenic and very likely responsible for the severe POI phenotype.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients

The proband presented at our fertility clinic for oocyte donation at the age of 35. She had been diagnosed with idiopathic POI at the age of 17 as she showed no pubertal development and PA. FSH and estradiol levels were respectively of 106 IU/L and <12 ng/L. At the time of diagnosis, she underwent a laparoscopic investigation which showed a small uterus and streak ovaries. Her puberty was induced with hormone replacement therapy. The patient did not present any dysmorphic features, her height was 163 cm with a BMI of 19 kg/m<sup>2</sup>. Standard karyotype and array CGH were normal. Her parents were Belgian and non-consanguineous. Her mother was menopausal at the age of 51 and the older sister presented the same

POI phenotype with PA and induced puberty. No other history of POI was reported in the family. However two paternal grandparent's sisters as well as a maternal aunt were unable to conceive without any reported underlying cause of infertility.

## 2.2 | DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes using Promega Maxwell<sup>®</sup> 16 Blood DNA Purification Kit AS1010 for WES and Promega Maxwell<sup>®</sup> RSC Whole Blood DNA Kit AS1520 for Sanger sequencing. Two mcg of DNA at a concentration  $\geq 30$  ng/ $\mu$ l was needed for WES.

## 2.3 | Whole exome sequencing

WES was performed in trio (the proband and her parents) in a paired-end 125 bp run on a HiSeq2000 Illumina sequencer at the Brussels Interuniversity Genomics High Throughput core (BRIGHTcore; <http://www.ngs.brighthouse.brussels/>). Exome capture was performed using Roche SeqCap EZ Human Exome Library v3.0 (64 Mb). Reads (FASTQ files) were aligned to the human reference genome GRCh37/hg19 using BWA algorithm version 0.7.10. Duplicated reads were then marked using Picard version 1.97. Alignment quality was improved using the GATK realigner and base recalibrator version 3.3. Variant calling was performed using GATK Haplotype Caller version 3.3. Variants' annotation and filtering were performed using the Highlander software (<http://sites.uclouvain.be/highlander/>). Variants were filtered for the following criteria:

1. Quality criteria: pass GATK (Cibulskis et al., 2011) standard filter and read depth  $\geq 10$ .
2. Allelic frequency, based on the maximum minor allele frequency found in 1000G (<http://browser.1000genomes.org/>), Genome Aggregation database (gnomAD; <http://gnomad.broadinstitute.org/>) and our NGS in-house database including more than 6125 individuals.
3. Functional impact: nonsynonymous or splice junction effect, using `snpeff_effect` from SnpEff (Cingolani et al., 2012).
4. Zygosity: compound heterozygous or homozygous variants.

Variant classification was based on the American College of Medical Genetics and Genomics (ACMG) variant interpretation guidelines of 2015 (Richards et al., 2015).

In order to exclude variants of other genes implicated in POI, we further performed analysis of a panel of 30 other POI causal and/or candidate genes in the proband. The complete list of the genes included in this panel has been described previously (Sassi et al., 2020).

## 2.4 | Sanger sequencing

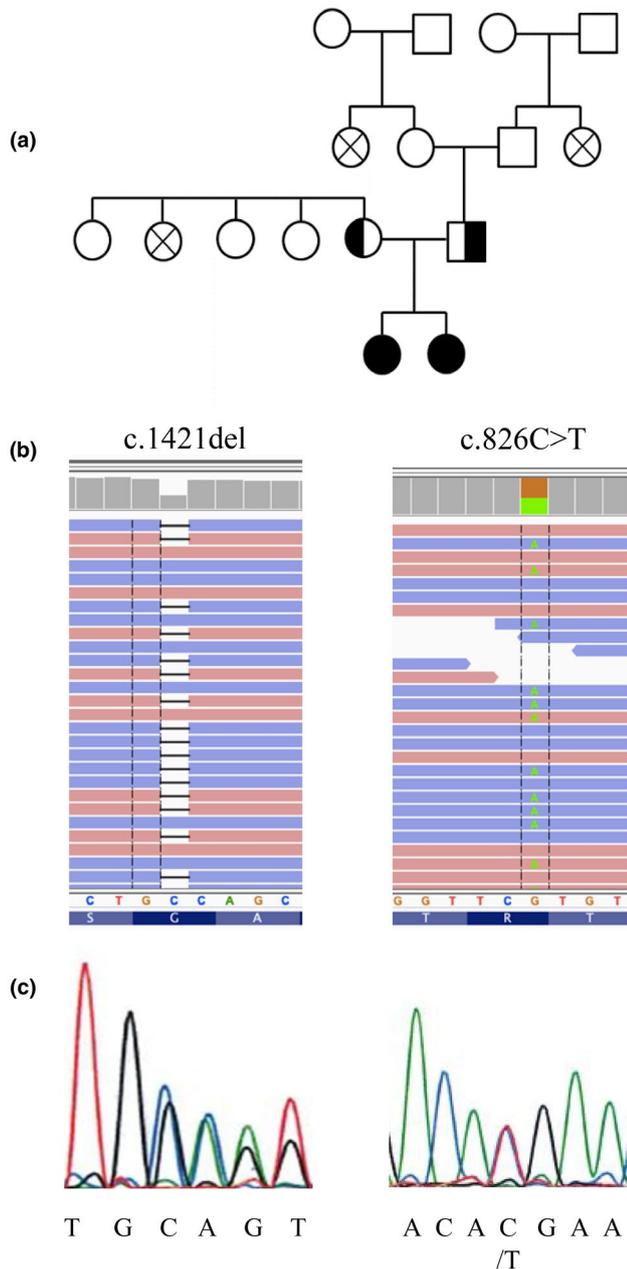
Sanger sequencing was performed for the validation of NGS results in the affected sister and their parents. DNA was amplified using a standard Polymerase Chain Reaction (PCR). PCR products were purified with BigDye XTerminator<sup>™</sup> Purification Kit (Applied Biosystems, Thermo Fischer Scientific), and analyzed on a 3130XL Genetic Analyser (Applied Biosystem). The Genbank reference sequence and version number of the sequenced gene (*NOBOX*) are respectively NM\_001080413 and NM\_001080413.3.

## 2.5 | Database submission

Identified variants have been submitted to LOVD database and following URL have been attributed: <https://databases.lovd.nl/shared/variants/0000708291>; <https://databases.lovd.nl/shared/variants/0000708292>

## 3 | RESULTS

WES identified two novel heterozygous *NOBOX* truncating mutations in the proband: c.1421del (p.(Gly474AlafsTer76)) inherited from the mother and c.826C>T (p.(Arg276Ter)) inherited from the father. Sanger sequencing confirmed that the sister also carried both mutations in a compound heterozygous state (Figure 1). The c.1421del was located in exon 8 and was predicted to induce a frameshift and a premature codon stop at amino acid residue 549. The c.826C>T was located in exon 4 and induced a premature stop codon predicting a truncated protein devoided of the large part of its homeodomain. These two variants have been reported neither in external nor in our in-house NGS databases. Based on the American College of Medical Genetics and Genomics (ACMG) variant interpretation guidelines 2015 (Richards et al., 2015), both variants were classified as pathogenic (c.826C>T (PVS1, PM2, PP3), c.1421del (PVS1, PM2, PM3)). Therefore they are very likely responsible for the severe POI phenotype in the two sisters. No other relevant variants were identified after variants filtering in the WES, nor in the 30 other genes included in the POI gene panel.



**FIGURE 1** Family segregation of *NOBOX* mutations. (a) Pedigree of the patient's family. Crossed circles refer to women who never conceived. (b) WES performed in the proband and her two parents identified 2 novel compound heterozygous *NOBOX* truncating mutations in the patient: c.826C>T (p.(Arg276Ter)) inherited from the father and c.1421delG (p.(Gly474AlafsTer76)) inherited from the mother. Both parents were heterozygous carriers of one mutation. The Genbank reference sequence and version number of the sequenced gene (*NOBOX*) are respectively NM\_001080413 and NM\_001080413.3. (c) Sanger sequencing in proband's affected sister showing the presence of both mutations

## 4 | DISCUSSION

We report here two novel truncating mutations in the *NOBOX* gene in two sisters presenting pubertal delay and primary amenorrhea with hypergonadotropic

hypogonadism. Both sisters were compound heterozygotes and had inherited a mutated allele from each parent.

*NOBOX* gene contains 10 exons encoding for an ovarian specific transcription factor expressed in primordial germ cells, oocytes and granulosa cells which plays a key role in the process of folliculogenesis (Bouilly et al., 2014; Lim et al., 2019; Rajkovic et al., 2004). *Nobox* knockout female mice are sterile due to a severe alteration of the germ cell cyst breakdown resulting in a drastic impairment of the primordial follicles formation and subsequent follicular transition from primordial to primary follicles with an early postnatal loss of all oocytes (Lechowska et al., 2011; Rajkovic et al., 2004). In *Nobox*<sup>+/-</sup> mice, the process of oocyte separation leading to the breakdown of the cysts and the formation of primordial follicles does not differ from the events occurring in wild-type mice (Lechowska et al., 2011).

The *NOBOX* protein contains a homeodomain able to directly regulate the expression of a series of genes playing key roles in the process of folliculogenesis such as *GDF9* (OMIM number: \*601918), *BMP15* (OMIM number: \*300247), *POU5F1* (OMIM number: \*164177), *KITLG* (OMIM number: \*184745), *FOXL2* (OMIM number: \*605597) and more recently shown, *RSPO2* (OMIM number: \*610575; Bouilly et al., 2014; Choi & Rajkovic, 2016; Lim & Choi, 2012; Qin et al., 2007; Rajkovic et al., 2004). The central role of *NOBOX* in the process of folliculogenesis together with the phenotype of ovarian insufficiency in mice disrupted for *NOBOX* made it a key candidate gene in women with non-syndromic POI. *NOBOX* variants have been consistently identified at the heterozygous state in cohorts of Caucasian and African POI patients, with a prevalence reaching 6.5% (Bouali et al., 2016; Bouilly et al., 2011, 2017; Ferrari et al., 2016). Fifteen *NOBOX* variants have been identified so far in POI patients, the large majority of them at the heterozygous state (Table 1). Two heterozygous large chromosomal deletions containing several genes including *NOBOX* and *CNTNAP2* (OMIM number: \*604569) were associated with PA and autism spectrum disorders (Bouali et al., 2016; Rossi et al., 2008) and it was suggested that haploinsufficiency of both these genes was sufficient for autism development and occurrence of primary amenorrhea (Bouali et al., 2016). In sporadic POI, until recently, the potential implication of heterozygous *NOBOX* variants was suggested to be related to *NOBOX* haploinsufficiency (Bouilly et al., 2017). More recently, functional studies of *NOBOX* variants identified in POI patients supported a dominant negative effect of mutated proteins at the heterozygous state (Bouilly et al., 2011). In fact, *NOBOX* gene mutations showed a defective transcriptional activity, an impairment of nuclear localization of the mutated protein, an intracellular aggregation suggestive of protein instability and a subsequent cell

TABLE 1 Previously reported NOBOX mutations and chromosomal deletions including NOBOX associated with POI

NOBOX variants (cDNA, protein)	Exon (No)	Zygosity	Mutation effect	POI phenotype	References	f (%)	FS	ACGM classification
c.271G>T, p.Gly91Trp	3	HZ	Missense	PA+delayed puberty SA (1 patient at 22years <sup>a</sup> )	33,40 35	0.214	Defects in NOBOX transcriptional activity, autophagosomal degradation, nuclear localization and protein stability.	Benign
c.349C>T, p.Arg117Trp	4	HZ Ho	Missense	PA+delayed puberty, PA, SA PA, SA	33, 35, 40 35	7.66	Defects in NOBOX transcriptional activity.	Benign
c.331G>A, p.Gly111Arg	4	HZ	Missense	SA	34, 40	0.00638	Defects in NOBOX transcriptional activity, autophagosomal degradation, nuclear localization and protein stability <sup>c</sup>	Likely benign
c.454G>A, p.Gly152Arg	4	HZ	Missense	Early menopause	34	0.331	Defects in NOBOX transcriptional activity, nuclear localization and protein stability <sup>c</sup>	Benign
c.567del, p.Thr190HisfsTer13	4	Ho	Frameshift	PA	38	0	Defects in NOBOX transcriptional activity.	Pathogenic
c.1025G>C, p.Ser342Thr	5	HZ	Missense	SA	33	0.000402	Defects in NOBOX transcriptional activity.	Uncertain significance
c.907C>T, p.Arg303Ter	5	HZ	Nonsense	SA	33	0.000401	Defects in NOBOX transcriptional activity.	Pathogenic
c.1064G>A, p.Arg355His	6	HZ	Missense	SA	29	0.11	Defect in binding capacity of Nobox homeodomain to NBE and negative dominant effect on the wilt-type protein.	Uncertain significance
c.1048G>T, p.Val350Leu	6	HZ	Missense	SA	33	0	Defects in NOBOX transcriptional activity.	Pathogenic
c.1112A>C, p.Lys371Thr	6	HZ	Missense	SA	40	0.195	Defects in NOBOX transcriptional activity.	Benign
c.1078C>T, p.(Arg360Ter)	6	Ho	Nonsense	no detail on POI phenotype	19	0.000403	Not performed	Pathogenic
c.1354G>A, p.Asp452Asn	8	HZ	Missense	PA, SA	34	1.1	Defects in NOBOX transcriptional activity, autophagosomal degradation, nuclear localization and protein stability <sup>c</sup>	Benign
c.1345C>T, p.Arg449Ter	8	HZ	Nonsense	SA	34	0.00251	Defects in NOBOX transcriptional activity, autophagosomal degradation, nuclear localization and protein stability <sup>c</sup>	Pathogenic
c.1489del, p.(Cys497ValfsTer53)	9	Ho	Frameshift	PA, incomplete PD	39	0.00287	Not performed.	Likely pathogenic
c.1856C>T, p.Pro619Leu	10	HZ	Missense	PA, SA (1 patient at 22 years <sup>a</sup> )	35, 40	0.111	Defects in NOBOX transcriptional activity	Benign
12 MB deletion including NOBOX	1->10	HZ	Large deletion	PA <sup>b</sup> , incomplete PD	36		Not performed	
12 MB deletion including NOBOX	1->10	HZ	Large deletion	PA <sup>b</sup>	37		Not performed	

Abbreviation: FS, functional study; f, GnomAD Exome allele frequency; HZ, heterozygous; HO, homozygous; NBE, NOBOX DNA-binding element; PD, pubertal development; PA, primary amenorrhea; SA, secondary amenorrhea.

<sup>a</sup>Same patient carrying variants (no precision if in cis or trans).

<sup>b</sup>Association to mental retardation, autism, seizures, dysmorphism and short stature.

<sup>c</sup>Variants which alter the FOXL2 transcriptional activity.

toxicity as well as a potential partial sequestration of wild type protein (Bouilly et al., 2011). This last mechanism is more consistent with mice models as no defect on primordial follicle formation has been observed in *Nobox*<sup>+/-</sup> mice (Lechowska et al., 2011).

We produce the first report of biallelic, compound heterozygous, truncating mutations of *NOBOX* in two outbred European sisters. Based on the ACGM guidelines, both mutations are pathogenic (Richards et al., 2015) and hence very likely to cause the ovarian phenotype. In silico data predicted that both mutations would induce truncated proteins. However, in the absence of RNA studies, we cannot exclude that both variants could be associated with a lack of protein as they were not located in the final exon of the gene. Both affected sisters presented a non-iatrogenic POI with a normal karyotype and a severe phenotype consisting of an absence of pubertal development at 17 years with PA.

Prior to our study, variants of *NOBOX* were reported at the homozygous state in four studies and much more frequently at the heterozygous state in several cohorts of POI patients (Table 1). Two homozygous mutations in the *NOBOX* gene were previously associated with PA (Li et al., 2017; Sehested et al., 2010). The homozygous c.1489del variant was identified in two Brazilian sisters with pubertal delay and PA in a consanguineous family (Li et al., 2017) while the homozygous truncating variant c.567del was identified in a Han Chinese patient (Sehested et al., 2010). Functional studies of this latter variant showed that the truncated *NOBOX* was associated with a decreased transactivation of a reporter gene and with the loss of *NOBOX* ability to induce G2/M arrest in transfected cell lines, suggesting an important role for *NOBOX* in cell-cycle regulation (Sehested et al., 2010). This observation supported the involvement of *NOBOX* in the regulation of germinal vesicle (GV) stage arrest in meiotic oocytes which extends from fetal life until adulthood (Sehested et al., 2010). Notwithstanding, one homozygous variant in *NOBOX* (c.349C>T) was identified in a patient with secondary amenorrhea but no additional clinical details were provided about this patient (Ferrari et al., 2016). More recently, a fourth homozygous mutation has been reported in *NOBOX* (c.1078C>T) in a patient with POI although it was not specified if she suffered from pubertal delay, primary amenorrhea or secondary amenorrhea (França et al., 2017).

Several heterozygous variants of *NOBOX* were identified in cohorts of patients presenting POI. In general, there was no consistent correlation between genotype and the clinical presentation: heterozygous mutations in *NOBOX* were mostly reported in patients with secondary amenorrhea although in some cases they were also identified in patients with PA with absent or incomplete pubertal development (Bouilly et al., 2011, 2015, 2017; Ferrari

et al., 2016). Similar *NOBOX* mutations were also associated with different phenotypes ranging from PA with incomplete pubertal development to SA, suggesting variable expressivity and a complex genetic background in which other susceptibility genes may worsen the phenotype leading to an earlier disease onset (Bouilly et al., 2016). So far, four heterozygous missense *NOBOX* mutations were associated with phenotypes encompassing PA and SA (c.271G>T, c.349C>T, c.1354G>A and c.1856C>T; Table 1).

It is noteworthy that only 5 out of the 15 reported *NOBOX* variants were classified as pathogenic or likely pathogenic based on the ACGM guidelines (Richards et al., 2015; Table 1). Although the variant in exon 10 (c.1856C>T) has been previously associated with POI at the heterozygous state suggesting that the C terminus part of the protein could also be involved in the transcriptional activity of *NOBOX*, the c.1421del variant located in exon 8 and inherited from the mother in our family seemed insufficient to induce POI at the heterozygous state as she was menopausal at the age of 51. This observation also suggests that haploinsufficiency of *NOBOX* in human could be tolerated as reported in mice models (Lechowska et al., 2011). Two paternal grandparent's sisters as well as a maternal aunt were unable to conceive without any reported underlying cause of infertility. However, we could not obtain further information about their age at menopause.

Our findings suggest that compound heterozygosity for the two *NOBOX* mutations is the cause for the severe POI phenotype in the two sisters.

In conclusion, we identified two novel compound heterozygous mutations of *NOBOX* in two sisters with PA and hypergonadotropic hypogonadism. Our findings enlarge the mutational spectrum of *NOBOX* and expand its contribution to the development of POI.

## ETHICAL COMPLIANCE

The study was approved by the Ethics committee of Erasme Hospital (study registration number: P2016/196/CCB B406201628264). The patient and her relatives gave their informed consent to be tested for a genetic etiology of POI.

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## CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## AUTHOR CONTRIBUTION

A.S: conception and design, acquisition, analysis and interpretation of data, drafting the article. J.D: conception and design, critical revision of the manuscript. S.D: acquisition, analysis, interpretation of data and revision of the manuscript. J.S: analysis, interpretation of data and revision of the manuscript. S.V: substantial contributions to conception and design, revision of the manuscript. M.L.B: substantial contributions to conception and design, revision of the manuscript. M.A: substantial contributions to conception and design, critical revision of the manuscript. A.D: conception and design, acquisition of the data, analysis and interpretation of the data, critical revising for important intellectual content. All authors gave final approval of the version to be published.

## DATA AVAILABILITY STATEMENT

The data that support this study is available upon a justified request.

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